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The Search for Guanosine Tetraphosphate (ppGpp) and Other Unusual Nucleotides in Eucaryotes†

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It came like magic in a pint bottle; it was not ecstasy but it was comfort.

Charles Dickens

INTRODUCTION

Bacteria possess the ability to adjust a number of diverse cellular processes rapidly in response to the availability of amino acids and other nutrients. Thus, starving an amino acid auxotroph for its required amino acid results in the so-called stringent response, consisting of the restriction of transcription of ribosomal ribonucleic acid (rRNA) and transfer RNA (tRNA), carbohydrate synthesis, phospholipid synthesis, lipid synthesis, phosphorylation of glycolytic intermediates, de novo nucleotide synthesis, transport of nucleobases and glycosides, and polyamine synthesis in addition to an increased rate of protein decay (see Cashel [11] for review). The bacterial cell emerges as an extremely efficient and complex organism capable of coordinating a network of interconnecting, yet biochemically distinct, biosynthetic pathways.

In 1969, Cashel and Gallant discovered two spots on autoradiograms from chromatograms of $^{32}PO_4$-labeled *Escherichia coli* extracts, which they called magic spots I and II (12). These substances were synthesized by stringent (*rel*$^+$) strains of *E. coli* in response to amino acid starvation. Isogenic relaxed (*rel*) strains upon amino acid starvation failed to induce the synthesis of these compounds and failed to give a stringent response. Magic spots I and II have since been identified as guanosine 5'-diphosphate 3'-diphosphate (ppGpp) and guanosine 5'-triphosphate 3'-diphosphate (pppGpp), respectively (see Lipmann and Sy [53] for review). The *relA* allele codes for stringent factor, a ribosome-associated protein that synthesizes ppGpp (7). The requirements for the ribosome-dependent synthesis of ppGpp and pppGpp are the following: stringent factor, both ribosomal subunits, messenger RNA (mRNA) and uncharged tRNA specific for the codon at the *A* site, adenosine 5'-triphosphate (pppA), and guanosine 5'-diphosphate (ppG) or pppG (7).

Several lines of evidence implicate ppGpp as the pleiotypic mediator of the stringent response. For example, the accumulation of ppGpp occurs within seconds after imposing an amino acid deficiency. This precedes the inhibition of rRNA synthesis by about 1 min (10). Fiil et al. (18), in an elegant set of experiments with modifier genes, have proved that it is not the *relA* gene product itself that determines stringency, but rather it is the concentration of ppGpp. In addition, in vitro experiments have directly implicated ppGpp as the negative effector of the syntheses of rRNA (6, 74), ribosomal proteins, elongation factors G and Tu, and RNA polymerase (nucleosidetriphosphate:RNA nucleotidyl-

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transferase, EC 2.7.7.6) subunit α (13, 52).

The stringent response is a remarkable device that bacteria have evolved to coordinate growth-related processes with the protein synthetic capacity of the cell. The relaxed bacterium may be analogous to a cancer cell in that neither can regulate its growth when common sense dictates that it should do so. Thus, the stringent response may be viewed as a model for the control of growth, which may or may not tell us something about how cellular growth in higher organisms is regulated. To determine if such a mechanism exists in eucaryotes, many investigators have attempted to ascertain whether guanosine polyphosphates or other unusual highly phosphorylated nucleotides are present in organisms other than procaryotes.

Before analyzing the literature, a few points concerning nucleotide hunting expeditions should be considered. Gallant and Margason (24) pointed out the hazards of identifying nucleotides solely on the basis of one-dimensional chromatography, a warning sometimes ignored by researchers in the field. They found an adenine-containing nucleotide in *Bacillus subtilis* that comigrated with ppGpp in one dimension, using KH_2PO_4, but was resolved from ppGpp in other chromatographic systems, as others have also found (55). In addition, condensed inorganic phosphate or polyphosphate is ubiquitous in nature (32) and can easily be mistaken for nucleotides in one-dimensional chromatograms of $^{32}PO_4$-labeled cell extracts. Also, contamination of eucaryotic cells with bacteria or mycoplasmas must be rigorously avoided. The stability of nucleoside polyphosphates on polyethyleneimine, a frequently used thin-layer ion exchanger, is poor. It is known that 20 to 30% degradation of ppGpp is routinely found. Loewen (55) found an adenine derivative that migrates near ppGpp on polyethyleneimine that is completely degraded.

In vivo assays for ppGpp usually consist of culturing the organisms or cells in $^{32}PO_4$, extraction with acid (usually formic acid), and, finally, thin-layer chromatography of the extract. Various techniques have been used to attempt to induce ppGpp synthesis in vivo in eucaryotes. By analogy to bacteria, amino acid or nutrient starvation is frequently done.

Occurrence of Guanosine Tetraphosphate (ppGpp) or Other Unusual Nucleotides in Procaryotes

It is not the intention of this review to include a detailed discussion of unusual nucleotides in procaryotes; however, it seems appropriate to summarize this topic before reviewing the eucaryotes.

A diverse variety of procaryotes other than *E. coli* have been examined for the presence of ppGpp and other highly phosphorylated nucleotides. These include: the gram-positive bacteria *B. brevis* (98), *B. subtilis*, (21), and *B. stearothermophilus* (82); the nitrogen-fixing bacteria *Rhizobium japonicum* and *Klebsiella pneumoniae* (A. G. Atherly and P. R. Russell, unpublished data); and the photosynthetic bacteria *Anacystis nidulans* (1, 60), *Anabaenopsis circularis* (84), *Anabaena cylindrica* (1, 84), and *Rhodopseudomonas sphaeroides* (16). Although the presence of ppGpp has been established in all bacterial species tested, it cannot be concluded that ppGpp is synthesized by the same mechanism as or functions in a similar manner to that in *E. coli*. It should be noted that no reports exist as to the presence or absence of ppGpp in mycoplasmas.

Rhaese and co-workers (78–80) have detected several unusual nucleotides in *B. subtilis* that have been identified as derivatives of adenosine: adenosine tetraphosphate [pp(5′)A(3′)pp], ppp(5′)A(3′)pp, and ppp(5′)A(3′)ppp. A mutant unable to synthesize pppAppp is also unable to sporulate, but revertants of the mutant can both sporulate and synthesize pppAppp. *relA* mutants still sporulate, suggesting that ppGpp is not involved in sporulation. Rhaese and Groscurth (79) report that ppApp and pppApp, but not ppGpp, are synthesized by ribosomes prepared from sporulating *B. subtilis* cells, whereas ribosomes prepared from vegetative cells synthesize only ppGpp. The enzyme system responsible for ppApp synthesis is not yet characterized. These reports thus indicate that another unusual highly phosphorylated nucleotide may play a key role in gene expression in bacteria; however, independent verifications of these findings are necessary before we can confidently make this conclusion.

Gallant et al. (25) have discovered a compound that they call phantom spot, characterized as "a form of GTP [pppG] with a modification of the imidazole portion of the purine ring." Phantom spot decreases rapidly upon energy source downshift. The authors suggest that the compound is a positive effector of cellular processes, including RNA accumulation. The compound seemingly is adjusted to the overall rate of phosphorylation in the cell.

Oki et al. (65) reported that strains of *Actinomycetes* produce an enzyme, purine nucleotide pyrophosphotransferase, capable of synthesizing purine nucleotide 3′-diphosphates, e.g., pApp, ppApp, pppApp, pGpp, ppGpp, pppGpp, and inosine pentaphosphate (pppIpp). Of 825 strains of bacteria and fungi tested, only 5, all *Actinomycetes*, synthesized the enzyme. The en-

zyme apparently is excreted from the cells during growth. Its function is unknown at present.

Loewen (55) has reported the presence of two novel nucleotides, DSI and DSII, in *E. coli* and other bacteria. These compounds inhibit in vitro RNA synthesis by *E. coli* RNA polymerase (44). DSI was reported to consist of coenzyme A and glutathione; DSII is a coenzyme A dimer with two equivalents of glutamic acid.

THE SEARCH FOR ppGpp IN EUCARYOTES

Lower Eucaryotes

Buckel and Böck (9) have looked for ppGpp in amino acid-auxotrophic strains of the mold *Neurospora crassa* (*leu*) and the chlorococcal green alga *Ankistrodesmus braunii* (*arg*). In both strains, RNA synthesis is stringently coupled to protein synthesis. The organisms were labeled with $^{32}PO_4$ for various durations of amino acid starvation. Chromatograms of the cell extracts revealed no label in the region where ppGpp migrates. These findings were not altered by increasing the specific activity of the $^{32}PO_4$, increasing the duration of amino acid starvation, or using two-dimensional chromatography. In addition, Alberghina et al. (2) failed to detect ppGpp in *N. crassa* grown on a poor carbon source, glycerol. In a similar study (57), *Chlamydomonas reinhardi* failed to synthesize ppGpp in response to amino acid starvation of an auxotrophic strain or by addition of the arginine analog canavanine to a wild-type strain.

However, Heizmann and Howell (34) examined the same auxotrophic strain of *C. reinhardi* but used slightly different detection techniques and found significant amounts of ppGpp. Heizmann and Howell found that addition of carrier pppA and pppG to samples (as in McMahon and Langstroth [57]) obscured the presence of ppGpp. It is not known why this effect was observed. They convincingly demonstrated the presence of ppGpp, using several criteria: two-dimensional chromatographic comigration with *E. coli* ppGpp, resistance to periodate oxidation, presence of guanine, acid and base lability, and alkaline phosphatase (EC 3.1.3.1) sensitivity. They demonstrated a reciprocal correlation between ppGpp/pppG ratios and chloroplast rRNA synthesis and suggested the existence of a form of stringent control. This relationship was the same as that found in bacteria. Heizmann and Howell (34) did not demonstrate the source of ppGpp synthesis, but effects of cycloheximide on ppGpp synthesis suggest a ribosomal site. Whether it is cytoplasmic or chloroplast, or both, was not established.

ppGpp synthesis also has been reported in *Euglena*, by Heizmann (33). A culture of *Euglena* growing exponentially in the dark was suspended in a solution of 30 mM KCl to inhibit growth. The time-dependent induction of two spots, which migrate slower than pppG on polyethyleneimine-cellulose and which could be labeled with $^{32}PO_4$ or [^{3}H]guanosine, was demonstrated. However, no further characterization of these compounds was reported. Heizmann suggests that ppGpp could regulate rRNA synthesis in *Euglena* chloroplasts, but no direct evidence for this was reported.

Klein (45) has examined cultures of the cellular slime mold *Dictyostelium discoideum* for the presence of ppGpp. This organism, when in the amoeba phase of its life cycle, is induced to differentiate by starvation. The differentiation is characterized by profound morphological and biochemical changes. Amoebae prelabeled with $^{32}PO_4$ and growing exponentially in a medium containing 100 μg of penicillin and streptomycin per ml were centrifuged and suspended in a buffer of 2-*N*-morpholinoethanesulfonate in an attempt to induce ppGpp synthesis. One- and two-dimensional chromatograms of extracts from these cells revealed a labeled compound that comigrated with authentic ppGpp. The lability of the compound to phosphomonoesterase digestion and nitrous acid oxidation resembled that of bacterial ppGpp. Only starved cells synthesized the compound. No evidence was presented, however, indicating that the compound contained a guanine moiety. The compound may be an isomer of ppGpp or some other nucleoside polyphosphate. The addition of antibiotics reduces the possibility of bacterial contamination. Others, however, have been unable to repeat these findings (W. A. Haseltine and A. Jacobson, unpublished data, as cited by Jacobson and Lodish [41]).

In auxotrophic strains of *Saccharomyces cerevisiae*, removal of a required amino acid halts the net synthesis of RNA after a brief lag (85). Similarly, Shulman et al. (90) found that rRNA, mRNA, and tRNA syntheses in *S. cerevisiae* were inhibited by about 80, 25, and 20%, respectively, after amino acid starvation. This restriction of rRNA synthesis resembles the stringent control of rRNA in procaryotes.

Kudrna and Edlin (46) searched for ppGpp in growing and amino acid-starved *S. cerevisiae*, but none was found. The authors suggested that the mechanism of coupled RNA and protein synthesis in yeast is different from that in bacteria. In contrast to the previous report, Pao et al. (66) claim to have found ppGpp in *S. cerevisiae* subjected to heat shock. Subjecting cells to 38°C for 6 min induced synthesis of the compound. None was found at room temperature.

That heat shock was required to elicit synthesis of the compound may explain why Kudrna and Edlin (46) failed to detect it in yeast. Pao et al. (66) reported an absence of bacterial contamination in their yeast cultures. The yeast compound was identified as ppGpp by the following criteria: (i) comigration with authentic ppGpp in two separate two-dimensional chromatographic systems; (ii) adsorption to charcoal; (iii) insensitivity to periodate oxidation, indicating the esterification of the 2′ or 3′ position of the ribose moiety; (iv) alkaline hydrolysis at the same rate as that of ppGpp and producing the same breakdown products as those of ppGpp; (v) zinc-activated inorganic pyrophosphatase (EC 3.6.1.1) yielding the same breakdown products as those of ppGpp; (vi) oxytetracycline was used (2 mg/ml); and (vii) 3-phosphoglycerate phosphokinase (EC 2.7.2.3), which phosphorylates ppGpp to pppGpp in vitro, also phosphorylated the yeast compound to a substance that comigrates with pppGpp. This evidence is the most complete characterization of ppGpp in any of the studies involving eucaryotes. Provided that the bacterial contamination tests were adequate (these were: plating on MacConkey or rich nutrient agar plates), the synthesis of ppGpp in yeast subjected to heat shock is well substantiated. Pao et al. suggest that the ppGpp present is synthesized in the mitochondria, although they give no direct evidence for this. In *E. coli*, oxytetracycline inhibits the nonribosomal synthesis of ppGpp in addition to the ribosome-dependent reaction (92). Therefore, nothing can be concluded about the site of ppGpp synthesis in yeast. Synthesis of ppGpp in yeast is not stimulated by deacylated tRNA, since heat shock in a strain of yeast with a temperature-sensitive isoleucyl-tRNA synthetase (EC 6.1.1.5) produced no more ppGpp than did the parent wild-type strain (66).

Higher Eucaryotes

The stringent response in bacteria has been compared to the control of growth in higher organisms. Hershko et al. (36) made a survey of the literature and concluded that, in mammalian cells, a diverse set of growth-related biochemical pathways and processes seems to respond in a coordinate manner to environmental conditions that restrict growth. The authors called this regulatory program the pleiotypic response. Imposing serum starvation on BALB/c 3T3 cells, a line of untransformed mouse fibroblasts, resulted in the inhibition of net RNA and protein synthesis, the stimulation of protein degradation, and the inhibition of uptake of glucose and of certain nucleic acid precursors. 3T3 cells transformed with simian virus 40 showed a much weaker response to serum starvation. Stanners and Thompson (96), however, have shown, using a line of Chinese hamster ovary (CHO) cells temperature sensitive for leucyl-tRNA synthetase (EC 6.1.1.4), that there was no coupling of protein synthesis to RNA synthesis and normal protein degradation at the nonpermissive temperature (two parameters of the so-called pleiotypic response). Despite similar contradictions, the possibility that ppGpp or a similar molecule might control growth in mammalian cells prompted several studies on the existence of such compounds in higher organisms.

Smulson (95) was the first to publish an attempt to detect ppGpp in eucaryotic cells. $^{32}PO_4$-labeled HeLa cells were amino acid starved by incubation in the absence of amino acids or with the isoleucine analog *O*-methylthreonine. Neither treatment induced detectable levels of ppGpp.

Tomkins and co-workers examined 3T3 cells subjected to various nutritional downshifts and were unable to detect any ppGpp synthesis (59). Cells were grown in $^{32}PO_4$ for 15 h before starvation for serum alone or for amino acids and serum. Formic acid extracts of these cells revealed no label in the region where ppGpp migrates on two-dimensional chromatograms. However, a mysterious spot, which the authors referred to as "x," migrated near pppG. Spot x disappeared during serum starvation and reappeared upon addition of either serum or insulin (both of which antagonize the pleiotypic response). No steps to identify this substance were reported, nor could we find any confirming reports. Tomkins and co-workers reported a limit of detection for ppGpp at 0.5% of the pppG level.

Fan et al. (17) examined a proline-requiring line of CHO cells and were unable to detect unusual nucleotides during normal growth or during amino acid or serum starvation. Similarly, Stanners and Thompson (96) in collaboration with J. D. Friesen failed to detect ppGpp synthesis in CHO cells temperature sensitive for leucyl-tRNA synthetase at the nonpermissive temperature. Also, Thammana et al. (100) induced isoleucine starvation in synchronized 3T3 cells by using a medium lacking the amino acid or a medium containing *O*-methylthreonine, an inhibitor of isoleucyl-tRNA synthetase. Extracts of cells from the beginning of the S phase into G2 were analyzed. No ppGpp was detected, regardless of the concentration of *O*-methylthreonine or the length of $^{32}PO_4$ labeling. Primary cultures of mouse embryonic fibroblasts gave the same negative results. Their limit of detection for ppGpp was placed at 0.5 to 1% of the counts in pppG.

Rapaport and Bucher have looked for ppGpp in normal and regenerating rat livers by injection of rats with $^{32}PO_4$, [^{3}H]hypoxanthine, or [^{3}H]-guanosine (70). Frozen livers were acid extracted, and the nucleotides were separated on a diethyl amino ethyl–Sephadex A-25 column in 7 M urea. Despite a high level of labeling of the nucleotide pool, no ppGpp or pppGpp was detected.

Embryonic material also has been examined for ppGpp synthesis. Irr et al. (40), using the ribosome-dependent ppGpp assay worked out for *E. coli* ribosomes, claimed to have found that ribosomes from 10- and 11-day mouse embryos are capable of synthesizing ppGpp, whereas ribosomes isolated from older embryos or from adult mouse livers were inactive. The compound synthesized by mouse embryonic ribosomes comigrated with authentic ppGpp in three different chromatographic systems and was labeled with either [α-^{32}P]pppG or [^{3}H]pppG. The amounts of ppGpp synthesis reported were significant, 37 to 69% of the rate found with *E. coli* ribosomes. However, Silverman and Atherly (91) have repeated these experiments with the same mouse strain without success. Ribosomes isolated from 10- to 13-day embryos were tested for ppGpp synthesis, and none was found. *E. coli* ribosomal high-salt wash, a source of stringent factor, was then added to the embryonic ribosomes in an attempt to stimulate the stringent factor. The results varied from minimal stimulation of ppGpp synthesis to no stimulation. It was concluded that mouse embryonic ribosomes alone are incapable of synthesizing ppGpp and that the lack of significant stimulation of *E. coli* stringent factor by the embryonic ribosomes indicated that it was unlikely that a ppGpp-synthesizing factor dissociated from the ribosomes during isolation. The latter conclusion gains support from the fact that the supernatant from the 100,000 × *g* centrifugation of 11-day-embryo extracts failed to stimulate the embryonic ribosomes to synthesize ppGpp. In addition, Silverman and Atherly demonstrated the absence of detectable ppGpp in acid-soluble pools isolated from mouse embryos of various stages cultured in the presence of $^{32}PO_4$. Recently, others also have reported an absence of ppGpp synthesis by ribosomes isolated from mouse embryos (63, 68).

Stimulation of ppGpp synthesis by using eucaryotic ribosomes and *E. coli* stringent factor has been attempted to establish whether the potential for ppGpp exists on the eucaryotic ribosomes. Pollard and Parker (68) found that addition of *E. coli* stringent factor to mouse embryonic ribosomes or to eucaryotic ribosomes isolated from a host of different organisms and tissues resulted in ppGpp synthesis. This finding is in apparent conflict with other reports. Failures to stimulate *E. coli* stringent factor with eucaryotic ribosomes have been reported for Ehrlich ascites (5), yeast cytoplasm, reticulocytes, calf brains (81), and mouse embryos (63). Slight stimulation of stringent factor, however, has been reported for ribosomes isolated from wheat germ (5) and yeast mitochondria (81), and abundant stimulation has been reported for *C. reinhardi* chloroplast ribosomes, but not cytoplasmic ribosomes (98). The latter two reports are discussed in greater detail in the following section. The reasons for these discrepancies remain unresolved, but they may involve the degree of purity of stringent factor and the ratios of stringent factor to ribosome used. The stimulation reported by Pollard and Parker (68) may be nonspecific, because *E. coli* stringent factor in the absence of ribosomes may be stimulated to synthesize ppGpp by addition of 20% methanol (99). The initial observation of Irr et al. (40) may have been due to bacterial ribosome contamination in their embryonic ribosome preparations.

No ppGpp synthesis was detected in vivo in sea urchin eggs or embryos in two studies (8, 67). In the former study, ribosomes isolated from sea urchin embryos failed to synthesize ppGpp in vitro.

In contrast to the preceding negative evidence, Rhaese (77) claimed to have found ppGpp and other nucleoside polyphosphates in various mammalian cell lines. The only autoradiograms presented, however, were of one-dimensional chromatograms of extracts from $^{32}PO_4$-labeled cells. No further characterization of these compounds was presented. It is well known that mammalian cells synthesize condensed inorganic phosphates (30) and other slow-migrative compounds (28), which could account for Rhaese's spots. Others have examined the same cell lines used by Rhaese and have not found ppGpp (17, 96, 100; R. A. Lazzarini, unpublished data, as cited by Pao et al. [66]; R. S. Esworthy, unpublished data). Clearly, the weight of the evidence is not in Rhaese's favor. This is especially evident in view of the fact that Rhaese discovered his compounds by merely labeling growing cells with $^{32}PO_4$; i.e., no special treatments were required to induce the spots.

Although the evidence favors an absence of greater than about 0.1 to 1.0 μM ppGpp in mammalian cells, the possibility of very low levels, perhaps synthesized by mitochondria, has not been ruled out.

Mitochondria and Chloroplasts

Because there are some similarities between bacteria and mitochondria and chloroplasts (e.g.,

the susceptibility of protein synthesis to certain antibiotics), these organelles have been investigated as possible sources of ppGpp synthesis.

Horvath et al. (37) reported ppGpp and pppGpp synthesis in isolated rat liver mitochondria and pppGpp synthesis in isolated spinach chloroplasts, both incubated in the presence of $^{32}PO_4$. ppG greatly stimulated the production of these compounds. No other nucleoside diphosphate would substitute for ppG. Addition of ppA plus ppG, however, stimulated the synthesis of the compounds about twofold over that found with ppG alone. Unfortunately, the characterization of these compounds was limited to one-dimensional chromatography; therefore, their identities remain undetermined.

Sy et al. (98), using ribosomes isolated from *C. reinhardi*, demonstrated that neither the cytoplasmic nor the chloroplast ribosomes were capable of ppGpp synthesis. However, addition of *E. coli* stringent factor to the chloroplast ribosomes resulted in abundant ppGpp synthesis. No ppGpp synthesis was found when the cytoplasmic ribosomes plus stringent factor were used. Thiostrepton, which inhibits *E. coli* ribosome-dependent ppGpp synthesis, also inhibited the chloroplast ribosome plus stringent factor assay. Combining *E. coli* ribosome subunits with chloroplast subunits in the presence of stringent factor gave significant levels of ppGpp synthesis. The small cytoplasmic ribosomal subunit plus the large subunit of either the *E. coli* or the chloroplast ribosomes in the presence of stringent factor resulted in a much lower level of ppGpp synthesis. Stringent factor addition to combinations involving the large cytoplasmic subunit gave little, if any, ppGpp synthesis. Similarly, Richter (81) demonstrated that yeast mitochondrial ribosomes could slightly stimulate ppGpp synthesis by *E. coli* stringent factor. As mentioned, ribosomes isolated from yeast cytoplasm, reticulocytes, or calf brains failed to stimulate stringent factor. The possibility exists that mitochondria and chloroplasts may contain stringent-factor-like enzymes that are loosely associated with the ribosomes and thus are lost during ribosome isolation. Nonribosomal ppGpp synthesis in mitochondria also is a possibility. *B. brevis* contains a ribosome-independent guanosine 5′,3′-polyphosphate synthetase (97).

Summary of Literature Concerning the Search for ppGpp in Eucaryotes

In almost every instance where ppGpp was found, there is evidence to the contrary. Nevertheless, the following conclusions can be drawn from the available data. (i) Lower eucaryotes may synthesize ppGpp in response to various environmental stresses (starvation, heat shock, etc.). (ii) Mammalian cells in culture probably do not synthesize ppGpp at levels higher than about 0.1 to 1.0 μM. (iii) Mouse embryos probably don't synthesize detectable quantities of ppGpp. (iv) Isolated eucaryotic ribosomes do not synthesize ppGpp. (v) Mitochondria and chloroplast syntheses of ppGpp and pppGpp have not been ruled out; if a ppGpp-synthesizing enzyme is present, however, it probably is either loosely associated with the ribosomes or not associated with the ribosomes at all.

Most of the research reviewed herein placed the limits of detection for ppGpp in the micromolar range. Nanomolar levels, if present, would have gone undetected. More sensitive methods (e.g., radioimmunoassay) will be required to determine if ppGpp is present in higher eucaryotes.

OTHER UNUSUAL NUCLEOTIDES IN EUCARYOTES

This section will be devoted to a class of unusual nucleotides or nucleotide-containing structures that may or may not be functionally related to ppGpp or to each other. Indeed, the only quality that these compounds can be convincingly said to share is that relatively little is known about them.

Diguanosine Tetraphosphate [G(5′)pppp(5′)G] and Related Structures

In 1963, Finamore and Warner (20) discovered a contaminant in RNA preparations from *Artemia salina* (brine shrimp) eggs. This compound was similar to ppG in its absorbance spectrum and in its ribose and phosphate content but differed from ppG in its breakdown products from phosphodiesterase I (EC 3.1.4.1) (20). The compound was extracted from 1,000 g of undeveloped brine shrimp eggs with sodium chloride and eluted from a Dowex 1-chloride column. The snake venom phosphodiesterase products of the compound were pG plus pppG. From these and other data, including paper chromatography, Finamore and Warner proposed the following structure: G(5′)pppp(5′)G. Subsequently, other unusual dinucleotides were found in brine shrimp eggs, namely, G(5′)ppp(5′)G (105) and, recently, G(5′)pp(5′)G and G(5′)ppp(5′)A (27). Diguanosine nucleotides have also been found in encysted embryos of the water flea, *Daphnia magna* (64), the fairy shrimp, *Eubranchipus vernalis* (109), and in the freshwater anostracan, *Branchipus stagnalis* (115). These compounds appear to be unique to organisms from the subclass *Branchiopoda* of the *Crustacea* and are not ubiquitous among crustacean species (111).

In dormant *Artemia* embryos, GppppG is present in extremely large amounts, comprising 45% of the ultraviolet-absorbing, acid-soluble material and 2% of the dry weight (27). G(5')ppp(5')G (109), G(5')pp(5')G, and G(5')ppp(5')A make up 7, 0.5, and 0.5%, respectively, of the ultraviolet-absorbing material in acid extracts of brine shrimp eggs (27).

These diguanosine compounds are stored and synthesized in the yolk platelets (110). GppppG synthetase (pppG:pppG guanylyltransferase), the enzyme responsible for the synthesis of GppppG, has been isolated from *Artemia* embryo yolk platelets (104). The enzyme catalyzes the synthesis of one equivalent of GppppG and inorganic pyrophosphate from two equivalents of pppG (108). Also, ppG and GppppG are converted to GpppG and pppG by the enzyme.

In *Artemia*, GppppG is synthesized very rapidly in the female ovarian tissue during oogenesis (109). It appears that the dinucleotide is unique to female brine shrimp (except for developing males) and is utilized during development (109).

Warner and Finamore sought to determine the role of these unusual nucleotides in brine shrimp development by studying nucleotide metabolism (107). At the time of hatching, the level of GppppG was found to drop rapidly, whereas the level of pppA increased fivefold during early development. The rise in pppA is significant in that *Artemia* species are defective in de novo purine biosynthesis, suggesting some sort of conversion of GppppG to pppA (14). Furthermore, it was demonstrated that although the level of total purines remained constant during early development, there was an increase in total adenine which equalled a decrease in total guanine (14). A pathway for the conversion of GppppG to pppA via the mixed dinucleotide A(5')pppp(5')G has been proposed based on the distribution of labeled precursors into pppA (103). The recent reports of G(5')ppp(5')A in *Artemia* cysts (27) and the formation of similar mixed dinucleotides from a back reaction of aminoacyl-tRNA synthetases (71) lends support to this hypothesis. Thus, GppppG apparently serves as a source of purines and phosphate bond energy during development (109). The dinucleotide structure may be necessary to protect the chemical bond energy in the molecule during dormancy (19). In this regard, GppppG synthetase is capable of catalyzing a reverse reaction, i.e., the synthesis of two equivalents of pppG from one equivalent of GppppG and inorganic pyrophosphate (108). In addition, an enzyme that degrades GppppG to pppG and pG (P^1,P^4-diguanosine 5'-tetraphosphate asymmetrical-pyrophosphohydrolase or diguanosinetetraphosphatase, EC 3.6.1.17) was discovered and characterized by Warner and Finamore (106). A more complete characterization of this enzyme and of a similar enzyme, dinucleosidase tetraphosphatase, from rat liver, has been reported (54, 101, 102).

Regulatory roles for GppppG have also been investigated. Finamore and Clegg noted that inhibition of deoxyribonucleic acid (DNA) synthesis in developing *Artemia* with 5-fluorodeoxyuridine prevented the large decrease in GppppG as well as the increase in pppA (19). They proposed that GppppG serves as a direct source of adenine for DNA synthesis. The utilization of GppppG may therefore control the onset of DNA synthesis in developing *Artemia*. The validity of this hypothesis rests on quantitative variations in nucleotide pools and on the distribution of labeled precursors ([^{14}C]pG, [^{14}C]pA and [^{14}C]pI) into DNA. Confirmation awaits a further characterization of the system.

Another point of regulation by GppppG involves GMP (pG) reductase (1.6.6.8) (75), which converts pG to pI. GppppG was found to be a specific positive effector of *Artemia* pG reductase. As mentioned, GppppG is hydrolyzed to pppG and pG by diguanosinetetraphosphatase. Conversion of the pG moiety of GppppG to pI may be a step in the conversion to pA (76).

Whether other events in the development of *Artemia* are regulated by GppppG and why these compounds appear to be unique to the subclass *Branchiopoda* of the *Crustacea* remain to be determined. Except for the absence of methylation, these compounds bear a resemblance to the capped ends of eucaryotic mRNA's: 7-methyl-G(5')ppp(5')*X* ... (87). This structure will promote initiation complex formation with ribosomes for most eucaryotic mRNA's and protects mRNA from 5' exonucleolytic digestion (23). This subject has been reviewed by Shatkin (87) and is outside the scope of this review.

Diadenosine Tetraphosphate [A(5')pppp(5')A] and Related Structures

Another dinucleotide, which is much more widespread in nature than GppppG, was discovered in 1966 by Zamecnik et al. (118). A(5')pppp(5')A was formed in a reaction mixture containing lysyl-tRNA synthetase (EC 6.1.1.6), lysine, pppA, and Mg^{2+}. A smaller amount of A(5')ppp(5')A was also formed. AppppA was presumed to be made by a back reaction of the first step in protein synthesis, namely, amino acid (aa) adenylation: $aa_1 \cdot pA \cdot$enzyme–aa_1 + pppA $\rightleftharpoons$ AppppA + aa_1 + enzyme–aa_1. Zamecnik et al. proposed that this back reaction would

serve as an "energy storage mechanism" during periods of protein synthesis slow down. A family of mixed dinucleoside tri- and tetraphosphates was formed by adding various nucleoside di- and triphosphates to the system (69). Tripolyphosphate and pppA have also been condensed to form pppp(5')A (116). In addition, A(5')ppp(5')Gpp and A(5')pppp(5')Gpp have been formed from ppGpp and pppGpp, respectively, by an *E. coli* lysyl-tRNA synthetase back reaction in vitro (71). A wheat embryo cell-free protein synthesizing system was also capable of forming these compounds.

Diadenosine compounds were demonstrated to be an in vivo phenomenon from the detection of AppppA in rat liver slices incubated with [^{14}C]adenine and in rat liver and muscle in vivo by intravenous injection of a rat with [^{14}C]adenine followed by analysis of tissue samples (116). AppppA levels were reported at 3×10^{-8} M in rat liver from the in vivo experiment and 6×10^{-7} M in the liver slices (116, 117).

Rapaport and Zamecnik have found AppppA in a variety of mammalian cell types (72). AppppA was labeled by the addition of [^{3}H]-adenine and $^{32}PO_4$ to cells in culture or by injection of the labeled precursors into mice. Purification of AppppA from the cell and tissue extracts was achieved by chromatography on a diethylaminoethyl-Sephadex A-25 column in 7 M urea followed by a diethylaminoethyl-cellulose–bicarbonate column and finally by polyethyleneimine–cellulose thin-layer chromatography. AppppA was found to be present in levels which reflected the degree of cellular proliferation. Human hepatoma cells grown in nude mice contained 0.8 to 1.2 μM AppppA, whereas normal mouse livers contained much lower levels of AppppA, 0.03 to 0.06 μM. Cells in culture ranged from 30 pmol of AppppA per mg of protein in rapidly growing cells to 0.15 mg of AppppA per mg of protein for slow-growing cells. Conditions which inhibit growth resulted in large decreases in AppppA. Serum or amino acid starvation resulted in a 30- to 50-fold decrease in AppppA. Inhibitors of protein or DNA synthesis caused even larger decreases in the AppppA concentration. The apparent correlation between the growth properties of cells and the level of AppppA and the metabolic lability and low concentration of AppppA led Rapaport and Zamecnik to propose that AppppA acts as a signal for a positive pleiotypic response.

The degradation of AppppA probably proceeds via the action of dinucleosidase tetraphosphatase, which degrades AppppA to pppA and pA (54, 101). The enzyme is specific for dinucleoside tetraphosphates and is similar to the diguanosinetetraphosphatase found in *Artemia* (101).

To ascertain the validity of assigning the term "pleiotypic activator" to AppppA, the following questions must be answered. (i) What are the kinetics of the AppppA decrease after serum and amino acid starvation? In other words, do the changes in AppppA levels precede or follow the parameters of the pleiotypic response? (ii) What are the molecular signals that regulate AppppA synthesis and degradation? (iii) What is the mechanism of action of AppppA in controlling growth?

Progress towards answering the last of these questions has recently been reported by Grummt (31). Addition of AppppA to baby hamster kidney (BHK) cells permeabilized by hypotonic treatment resulted in a stimulation of DNA synthesis in cells previously arrested in G_1 by serum starvation. Several inhibitors of DNA synthesis prevented AppppA stimulation of DNA synthesis; cycloheximide, however, had no effect. The stimulation was dependent on the concentration of AppppA and was abolished by digestion of AppppA with phosphodiesterase I. Alkaline phosphatase, which will not digest AppppA, had no effect. [^{3}H]AppppA had a half-life of about 40 min in the permeabilized cells. Replication eyes were scored by electron microscopy in the DNA isolated from permeabilized G_1-arrested cells incubated in the presence or absence of AppppA. The frequency of replication eyes was 20-fold higher in the DNA from AppppA-treated cells. The DNA synthesized in permeabilized G_1-arrested cells after treatment with AppppA was examined by using alkaline sucrose gradients. Pulse-labeled material had a sedimentation coefficient of 4S, which was chased into larger molecules. These experiments raise the possibility that AppppA may be a signal for the initiation of DNA synthesis. This intriguing hypothesis awaits confirmation and characterization.

Adenosine Tetraphosphate (ppppA) and Related Structures

In 1953, Marrian reported ppppA as a contaminant in pppA preparations from ox muscle (61, 62). Commercial pppA from yeast was also found to contain ppppA as well as pppppA (86). In addition, ppppA has been reported in horse muscle (51), rabbit muscle (94), in rat liver slices incubated with [^{14}C]adenine (116), and in isolated rat liver mitochondria incubated with $^{32}PO_4$ (35).

Small and Cooper isolated an enzyme from rabbit muscle which will hydrolyze ppppA to pppA and inorganic orthophosphate (93). The

hydrolase enzyme was not specific for ppppA, however, as ppppI was hydrolyzed to pppI and inorganic orthophosphate; and tripolyphosphate was converted to pyrophosphate and inorganic orthophosphate by the enzyme. ppppA has been reported to constitute 0.035% of the adenosine mononucleotide content of rabbit muscle (94). ppppA could be synthesized in vitro by yeast 3-phosphoglycerate kinase (EC 2.7.2.3): pppA + 1,3-diphosphoglycerate → 3-phosphoglycerate + ppppA (94). Rabbit muscle 3-phosphoglycerate kinase, however, was incapable of synthesizing ppppA. Therefore, this enzyme is not responsible for the ppppA found in rabbit muscle. Zamecnik and Stephenson have synthesized ppppA in vitro from tripolyphosphate and pppA in a back reaction of lysyl-tRNA synthetase (116).

The biological role, if any, for ppppA is not known. Some functions for the compound have been proposed. Winand-Devigne et al. (113) claimed that ppppA is capable of splitting carp muscle actomyosin into actin and myosin. However, the possibility that this splitting action occurred due to pppA formed by the hydrolysis of ppppA is difficult to rule out. A possible regulatory role for ppppA is indicated by the potent inhibition of dinucleosidase tetraphosphatase by ppppA as well as by ppppG (54, 101, 102). ppppG has been found in commercial pppG (26, 102).

The possibility that ppppA is synthesized during its isolation has been raised (94). High levels of pppA and calcium at pH 8.5 and 40°C resulted in the slow nonenzymatic synthesis of ppppA. The amounts of ppppA found in tissues make this explanation appear unlikely. Alternately, ppppA may be a degradation product of AppppA.

pppA(2′)p(5′)A(2′)p(5′)A

Studies on the antiviral agent interferon have resulted in the discovery of a particularly fascinating unusual oligonucleotide structure by Ian Kerr's group at the National Institute of Medical Research in London, England. Roberts et al. (83) have reported that the incubation of cell sap from interferon-treated mouse L cells with pppA and double-stranded RNA resulted in the synthesis of a heat-stable, low-molecular-weight inhibitor of in vitro protein synthesis. Hovanessian et al. (38) demonstrated that the enzyme responsible for the synthesis of the low-molecular-weight inhibitor could be purified by passage of the postribosomal supernatant from interferon-treated cells over a poly-inosine-cytosine–Sepharose column. Both the enzyme and a double-stranded-RNA-dependent protein kinase were retained on the column. The low-molecular-weight inhibitor was synthesized by incubation of the poly-inosine-cytosine-retained material with pppA and magnesium. Kerr and Brown (42) have elegantly demonstrated the structure of the low-molecular-weight inhibitor to be pppA(2′)p(5′)A(2′)p(5′)A. An oligomeric series of these compounds was discovered (42), from the dimer, pppA(2′)p(5′)A, to the pentamer, pppA[(2′)p(5′)A]$_4$, with traces of higher forms detectable. The main product of the reaction was pppA(2′)p(5′)A(2′)p(5′)A. This compound is effective in inhibiting in vitro protein synthesis at subnanomolar levels. Kerr et al. (43) determined that the level of pA in the inhibitor required to give a 50% inhibition of translation of encephalomyocarditis virus RNA in mouse L-cell extracts ranged from 0.3 to 1 nM. The inhibitory activities of the trimer, tetramer, and pentamer were similar; however, the dimer was only about 1/10 as active (42).

Williams and Kerr have recently demonstrated pppApApA to be a potent inhibitor of protein synthesis upon addition to BHK-21 cells made permeable by hypertonic medium (112). In the permeabilized cells, the bacterial alkaline phosphatase-resistant core, ApApA, had about 1/10 the inhibitory activity of the complete trimer, pppApApA. The core is inactive in cell-free systems, indicating the possibility of phosphorylation of the core in this system. None of the pppApApA compounds will inhibit viral protein synthesis upon addition to virus-infected cells (112).

Evidence is accumulating that the oligonucleotide inhibits protein synthesis by activating an mRNA-degrading nuclease. Clemens and Williams (15) demonstrated that pppApApA promotes the disaggregation of polysomes in lysates from reticulocytes or mouse L cells. This effect was not due to an inhibition of protein synthesis initiation with a subsequent runoff of ribosomes. When emetine was used to inhibit ribosome runoff, polysome breakdown was still enhanced by pppApApA. Partial purification of a ribonuclease that is indirectly activated, presumably via formation of pppApApA, has been reported (73). Double-stranded-RNA-, pppA-dependent nuclease activity was much greater in extracts from interferon-treated cells than in those from control cells. Baglioni et al. (3) studied the degradation of vesicular stomatitis virus mRNA in cell extracts from interferon-treated and control cells. Vesicular stomatitis virus RNA degradation was enhanced by pppApApA, although the level of nuclease activity was similar in the interferon-treated and control cell extracts. The

"pppApApA-activated nuclease" showed no specificity for viral or cellular mRNA or for free versus polysomal mRNA. Baglioni et al. propose that, in vivo, the nuclease could preferentially degrade viral RNA if pppApApA is synthesized on the viral replicative intermediate, thereby activating nuclease at the site of viral RNA synthesis. This theory is supported by the finding that pppApApA synthetase binds to and is activated by double-stranded RNA.

Interferon-induced inhibition of protein synthesis includes mechanisms other than the one reviewed herein (48, 119). Also, interferon-induced viral resistance probably involves cellular processes other than translation (see Friedman [22] for review).

Is pppApApA synthesized in cells not treated with interferon? Ball and White (4) reported that extracts from non-interferon-treated chicken embryo cells synthesize 0.2% of the amount of pppApApA synthesized by interferon-treated cells. The pppApApA synthetase evidently is induced by interferon, but low residual levels may be present in normal, untreated cells. Hovanessian and Kerr (39) have demonstrated the formation of the oligonucleotide inhibitor from rabbit reticulocyte lysates in response to double-stranded RNA and pppA and have purified the enzyme that synthesized the inhibitor by poly-inosine-cytosine–Sepharose chromatography. The compound synthesized seemed to be identical to that made from interferon-treated mouse L cells, as judged by chemical and biological characterization. These last two reports point to the possibility that pppApApA may play a role in the regulation of protein synthesis beyond that observed during viral infection. Also, pppApApA is unstable in cell-free systems from control or interferon-treated cells, indicating that a pathway for the degradation of this compound is prevalent (112). Whether this unusual oligonucleotide possesses other regulatory functions remains to be seen.

Unclassified Structures

There are other structures which may be considered to belong to this group of unusual nucleotides. For instance, a charcoal-extractable, acid-soluble inhibitor of rRNA synthesis has been reported in *Xenopus* embryos (89, 114). This substance has not yet been characterized, but it may be a nucleotide. The inhibitor has been reported to be synthesized in the terminal stage of oogenesis (88).

Rapaport and Bucher have discovered two adenine-derived nucleotides in rat livers labeled in vivo with $^{32}PO_4$, [^{3}H]hypoxanthine, or [^{3}H]-adenine (70). One compound, "A," is preferentially synthesized during liver regeneration and carries one charge more than pppA. The other compound, "B," carries an additional phosphate group and was present in normal and regenerating rat livers.

Also, Silverman and Atherly (91) have reported finding three phosphate-containing compounds (A1, A2, and B) in mouse blastocysts that migrate slower than pppG on polyethyleneimine–cellulose chromatograms.

The presence of diguanosine nucleotides, which were presumed to control transcription (58), was reported in fungi (47), but Goh and LeJohn have since retracted their original characterization of these compounds, which they refer to as HS-1, -2, and -3 (28). The absence of

TABLE 1. *Summary of unusual nucleotides and related structures in eucaryotes*

Nucleotide	Cell type or organism	Putative functions
pp(5′)G(3′)pp	See text	Regulation of rRNA synthesis and pleiotypic effects
G(5′)pppp(5′)G	*A. salina* and other organisms from the subclass *Branchiopoda* of the *Crustacea*	Storage form for purines, energy for development, possible regulatory roles in DNA synthesis and in nucleotide metabolism
A(5′)pppp(5′)A	Mammalian cells	Control of DNA synthesis, pleiotypic activator
pppp(5′)A	Yeast, mammalian muscle, rat liver, rat mitochondria	May split actomyosin, inhibits dinucleosidase tetraphosphatase
pppA(2′)p(5′)A(2′)p(5′)A	Mammalian and chicken embryo cells	Inhibition of protein synthesis by activation of ribonuclease(s), other regulatory roles possible
HS-1, HS-2, HS-3	Fungi, mammalian cells	Control of growth

diguanosine compounds in fungi was confirmed by Warner et al. (111). Goh et al. now claim that the HS-3 compound is a complex dinucleotide structure of glutamyl-ppA-ribitol-uridine 5'-diphosphate-mannitol-tetraphosphate (29). HS-3 is presumed to coordinate salvage and de novo nucleotide biosynthetic pathways in mammalian cells. Goh et al. (29) show a five- to sixfold increase in HS-3 upon withdrawal of L-glutamine (but not with isoleucine) from CHO cells. They estimate the HS-3 concentration at a minimum value of 120 μM after glutamine starvation (49). When HS-3 accumulated in CHO cells, DNA and RNA synthesis decreased, and vice versa. However, a cause-and-effect relationship is difficult to establish. In further studies, Lewis et al. (49, 50) show that HS-3 inhibits ribonucleoside-diphosphate reductase (EC 1.17.4.1) significantly (50 to 80%) at concentrations that correspond to intracellular levels after glutamine withdrawal. Ribonucleotide reductase has been suggested as a major control point in DNA synthesis (56). There will doubtlessly be other compounds found to add to the list.

SUMMARY

Table 1 summarizes some of these compounds and their putative functions. Although some are effective at subnanomolar quantities (e.g., pppApApA), others form a major constituent (GppppG). The diversity of function and structure of these unusual nucleotides in nature is becoming increasingly apparent. Research efforts directed in this field should provide exciting prospects for future endeavors.

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ADDENDUM IN PROOF

A recent report indicates that although mouse embryo or rabbit reticulocyte ribosomes fail to stimulate *E. coli* stringent factor to synthesize ppGpp, the ribosomal proteins prepared from these ribosomes did stimulate ppGpp synthesis by stringent factor (O. Martini and D. Richter, Mol. Gen. Genet. **166**:291-297, 1978).

Interferon-mediated inhibition of the initiation of protein synthesis by phosphorylation of the translation initiation factor eIF-2 was recently reported to be independent of the 2-5 A system. It was also reported that 2-5 A-activated nuclease inhibited the translation of Mengo virus RNA in an mRNA-dependent reticulocyte lysate to a greater extent than added globin mRNA. The authors of the report suggest that this may be due to the fact that the Mengo virus RNA is much longer than the globin RNA or to some "selectivity of interferon action" (P. J. Farrell, G. C. Sen, M. F. Dubois, L. Ratner, E. Slattery, and P. Lengyel, Proc. Natl. Acad. Sci. U.S.A. **75**:5893-5897, 1978).

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